IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	)	
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Gilbert GORR et al.	)	
	)	
Serial No. 10/089,450	)	Group Art Unit: 1638
	)	
Filed: March 29, 2002	)	Examiner: KUBELIK, Anne R.
	)	
For: METHOD FOR THE PRODUCTION OF PROTEINACEOUS SUBSTANCES	)	

DECLARATION UNDER 37 C.F.R. § 1.132 BY GUNTHER NEUHAUS

Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

1. I, Gunther Neuhaus, state that I am an expert in the field of plant cell cultivation research and development. I hold a PhD in Biology (Botany, Zoology) and am a professor of Cell Biology at the University of Freiburg. A copy of my Curriculum Vitae is attached herewith as evidence of my relevant expertise.

2. I am familiar with the above-captioned patent application and claims. In order to appreciate biological differences and predicted activities between cells isolated from vascular plants, such as tobacco cells and whole protonema tissue, in this declaration, I review relevant literature.

3. A bryophyte is a non-vascular plant, which generally means it does not have a vascular system (xylem and phloem). Bryophytes do not have flowers and do not

produce seeds. They have enclosed reproductive systems and reproduce via spores. The bryophyte life cycle progresses from spore to protonema to gametophore.

4. *Nicotania tobacum* is commonly referred to as the tobacco plant. Tobacco plants are vascular in nature. Vascular plants are distinguished in part by the presence of vascular tissues (xylem and phloem), which circulate resources throughout the plant. The tobacco plant produces seeds and flowers.

5. In intact plants, plant cells are surrounded by a cell wall. The cell wall is a tough rigid layer that protects plant cells from environmental conditions, which can be highly varied. Among these include light conditions such as content of UV or the broad variability in air humidity.

6. Although the cell wall provides a rigid outer barrier, plant cells are interconnected by plasmodesmata. Plasmodesmata are pores through the cell wall, by which individual cells may be interconnected by membranes, cytoplasm and by the endoplasmatic reticulum. This is essential for a vascular plant as there are cells without nuclei existing, which receive needed nutrients and expression products from neighboring cells (accompanying cells support the sieve elements by this method in the phloem).

7. There are different approaches to studying biological processes in plants. Among these include studying the intact plant itself and studying plant cells such as cell

suspensions derived from intact plants. Whether to perform experiments on intact plants or cell suspensions may depend on the process studied.

8. Many plant experiments are performed using cell suspensions. Among these including *Nicotania tobacum* clone-1 cells ("NT-1 cells") and Bright Yellow 2 cultivar of the tobacco plant ("BY2 cells"). In suspension NT-1 and BY2 cells float independently or in groups but the suspension itself is not interconnected by plasmodesmata as found in intact plants. While cell suspensions do not retain many of the characteristics of cells of an intact plant, many biological processes such as cell division, cytoskeleton and hormone signaling may be studied using cell suspensions.

9. Although cell suspensions such as NT-1 cells and BY2 cells are useful in studying biological processes such as those that affect cell division, the cytoskeleton and hormone signaling, experiments directed towards the cell wall itself including its function, its protective characteristics or its role as a barrier are not considered reflective of cells within the intact plant. This is due in part to the manipulation of the cell during the culturing process to achieve its specialized characteristics.

10. To better understand the manipulation of the plant cell during its transformation from intact plant to a cell suspension, I provide the following overview of the processes.

11. In vitro cell cultures from vascular plants are initiated from sterilized organ explants. These initial explants are induced to form an undifferentiated callus (cell mass). The cells in this callus lose typical plant and organ specific expression (such as expression of photosynthetic genes and several metabolic genes) and only express so called housekeeping genes. As such these callus cells also lose over time the potential for regenerating a complete plant. These cells are considered specialized cells and are so called "habituated plant cells."

12. To establish a suspension culture from the specialized callus cells, several steps have to be followed. First, the callus has to be broken down in small mostly single cell aggregates which have to be cultured in a highly complex liquid culture medium including vitamins, sugars as well as plant hormones. Afterwards the cell suspension has to be subcultured every 8 to 10 days to ensure continuous cell division. Upon this subculturing procedure the cells have to be sieved, so that mostly only single cells or at least small cells serve as starting culture for the next growing cycle. If this is not done in the appropriate way the cells will die in the old suspension culture upon nutrient deficiency. In addition if the cell aggregates are grown too big they also will die as the inner cell mass will not get the required nutrients.

13. There is a high heterogeneity in the starting plant cell suspensions. Further culturing is performed to eliminate this heterogeneity. Thus, the specialized cell suspension is adapted for liquid culture conditions. Among the adaptations, especially with respect to homogeneity in "humidity" there is no need for a strong barrier i.e. rigid

cell wall against the environment surrounding medium. In addition, cell suspensions such as NT-1 and BY2 cells have also been cultured over years and selected for additional special selected features (e.g. synchronized cell cycle in tobacco BY2 cell cultures).

14. Once specialized into NT-1 or By2 cell suspensions, structural changes in their cell biology appearance and in particular the cell wall is evident. For instance, whereas cells in whole intact vascular plants are interconnected by plasmodesmata, the suspension of NT-1 and BY2 cells are not interconnected. Instead, NT-1 and BY2 cells are typically found floating independently or in small groups.

15. Functionally, NT-1 and BY2 cells obtained from suspension compared to cells provided within the native intact vascular plant behave differently. This can be evidenced in part by taking plant cells from a cell suspension and culturing them on an agar medium under same conditions in which sterile in vitro plants or plant cuttings can be grown easily. Within one week all cells from the suspension culture stop their division capacity and due to their artificial nature caused by their culturing technique (in suspension) they will die after one week.

16. Since the barriers of NT-1 cells and BY2 cells are known to be manipulated to facilitate culturing in suspension, it would not be logical to study the role or characteristics of a plant cell wall using NT-1 cells or BY2 cells. Instead, one would logically study whole intact tissue or a whole intact plant. Thus, a comparison between

effects observed in a culture of suspended specialized cells would be difficult to transfer to an intact plant.

17. A comparison between the cultivation of differentiated bryophytes or differentiated tissue thereof in liquid culture, in which the differentiated non-vascular plant gametophyte or protonema is cultivated, and a suspension culture of specialized plant cells derived from vascular plants is scientifically very difficult. In one case the cultured material is the whole differentiated organism (bryophyte) or differentiated tissue thereof like protonema whereas in the other case a selected artificial and undifferentiated sporophytic cell suspension is used. The difference is mostly obvious when explants will be taken from both suspension cultures and plated on agar. As bryophyte material from suspension culture represents a whole organism or tissue thereof - in both cases the differentiated cells are highly regenerative - it will grow and develop gametophytic fully developed organisms, whereas a cell from the sporophytic cell suspension like BY2 or NT-1 cell cultures will perform in the ideal case one or two cell divisions and then slowly will die under these conditions, but never develop - even under the best nutrients - to a whole sporophytic organism.

18. There exists also in higher plants gametophytic cell suspension cultures which are able to regenerate to non fertile plants, but the culture conditions are very limited. First the explant material is immature gametophyte (microspores) just after meiosis, secondly these cultures are genetically very limited, as they have very low expression activity and thirdly the cultures have to be initiated for regeneration within

short time (1 – 2 month) as they lose their regeneration capacity very fast and develop into slow dividing and finally dying cells. Moreover the cell wall of this gametophytic plant material is different from that of all other vascular plant cells, as their normal development is primed to be a mature gametophyte, which is a pollen, that has to survive rough environmental conditions such as drought and high/low temperatures (this is made by deposition of lipophil material within the cell wall to avoid any loss of water and thereby also any secretion of water soluble compounds like salts into the surrounding media). By this the pollen (the gametophyte of vascular plants) contains the most thick and hardest cell wall that can be found in the plant kingdom.

19. In conclusion from a point of a cell biologist, a direct comparison between a moss suspension culture containing whole gametophyte or tissue thereof (like protonema) and a suspension of specialized cells from vascular plants like NT-1 and BY2 is therefore inadequate and biological conclusions -especially regarding the barrier e.g. cell wall system against the outer environment- are as useless for transfer to the whole gametophyte or tissue thereof (like protonema) of liquid bryophyte cultures and vice versa.

20. I declare under penalty of perjury that the foregoing is true and correct, that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code,

and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Signed by,

Date: 26.06.09

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Grades and Post Graduate Studies

1974	Gymnasium Linz / Austria

University

1974-1978	Study (Biology) at the University Salzburg (Austria) (Mag. rer. nat.)
1980	Graduation for Dr. phil. in Cell Biology and Botany (University Salzburg, Austria)
1993	Habilitation at the ETH-Zürich ("New approaches in plant development"), Venia legendi „Plant Developemnt"

PROFESSIONAL EXPERIENCE

1980	Research Assistant at the University Salzburg (Austria), Institute for Plant Sciences
1980-1982	Postdoc at the Max-Planck-Institute für Cell Biology, Ladenburg/Heidelberg, Germany
1982	6 month stay as Visiting Scientist at the Rockefeller University, New York with Prof. Dr. N.-H. Chua, USA
1982-1987	Research Assistant at the Max-Planck-Institute for Cell Biology, Ladenburg/Heidelberg, Germany
1987-1993	Assistant Professor at the Institute for Plant Sciences at

	the ETH-Zürich, Switzerland (Swiss Federal Institute of Technology)
1993-1996	Visiting Associate Professorship at the Rockefeller University, Department of Plant Molecular Biology, Prof. N.-H. Chua (New York, USA)
since 1995	Head of Department, Institute of Cell Biology, University Freiburg, Germany
since 1998	Managing Director oft he Center of Applied Sciences University Freiburg, Germany
2002	Founder of the Biotech Company "greenovation", Freiburg, Germany (together with Prof. Reski)
Since 2002	Member of the "BioValley Expert" Teams
2002	Member of the "Task force group in Life Science" of the University Freiburg
since 2003	Advisor "Biotechnology-Team Baden-Württemberg"
since 2003	Elected Member of the "Strasburg-Author team"

Awards

1980	1. Preis der Stadt Salzburg für die beste Doktorarbeit.

Elected Memberships

2000	Deutsche Akademie der Naturforscher „Leopoldina" (German Academy of Science)

Referee

For international Organizations DFG, NSF, USDA, Japanese Frontier Science Program, Swiss Nationalfonds, Human Science Frontier Program Organization, und other Organizations.

Journals - Plant Physiology, Plant Cell, Plant Cell Physiology, Cell, Nature, EMBO J., Science, MGG, Plant Cell & Environment, Plant Mol. Biology, Planta, etc.

Book Authorship:

Strasburger - Lehrbuch der Botanik: Bresinsky, A., **Körner**, C., **Kadereit**, J.W., **Neuhaus**, G., **Sonnewald**, U. 36. Aufl., 2008, XVI, 1176 S. 957 Abb., 465 in Farbe., Geb. ISBN: 978-3-8274-1455-7

Freiburg, 2.2.2009 ***Gunther Neuhaus***

Prof. Dr. Gunther Neuhaus

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